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Chicken Nkx-2.8: A Novel Homeobox Gene Expressed in Early Heart Progenitor Cells and Pharyngeal Pouch-2 and -3 Endoderm

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Members of the NK family of homeobox transcription factors regulate critical steps of organogenesis during vertebrate development. In the studies described in this report, we have isolated and functionally characterized the chicken *Nkx-2.8* (cNkx-2.8) cDNA and protein and defined the temporal and spatial pattern of *cNkx-2.8* gene expression during chicken development. *cNkx-2.8* transcripts are first detectable at HH stage 7 in the splanchnopleura. At stage 10$^+$, the *cNkx-2.8* gene is expressed in the linear heart tube and the dorsal half of the vitelline vein. However, after looping, HH stage 13, *cNkx-2.8* is no longer expressed in the looped heart tube, but is expressed in the ventral pharyngeal endoderm. At stage 15, in addition to the pharyngeal expression pattern, *cNkx-2.8* is expressed in the ectoderm of the pharyngeal arches and the aortic sac. By HH Stage 17, *cNkx-2.8* expression is detectable in lateral endoderm of the second and third pharyngeal pouches, the posterior portion of the aortic sac, and the sinus venosus. cNkx-2.8 binds to previously characterized Nkx2-1 and Nkx2-5 DNA-binding sites and overexpression of cNkx-2.8 transactivates a minimal promoter which contains multimerized Nkx-2 DNA-binding sites. In addition, cNkx-2.8 and serum response factor can coactivate a minimal cardiac α-actin promoter. These data are consistent with a model in which cNkx-2.8 performs a unique temporally and spatially restricted function in the developing embryonic heart and pharyngeal region. Moreover, the coexpression of *cNkx-2.5* and *-2.8* raises the possibility that cNkx-2.8 may have a redundant role with cNkx-2.5 in the coalescing heart tube and may play an important role in the transcriptional program(s) that underlies thymus formation. The existence of multiple NK-2 family members and their partially overlapping patterns of expression are discussed within the framework of a "Nkx code."

INTRODUCTION

Defining the molecular mechanisms underlying the establishment and maintenance of cardiac muscle differentiation requires the elucidation of lineage-restricted transcription factors that direct the appearance and commitment of cardiac primordial cells. Homeobox genes have been studied extensively in *Drosophila,* where they are involved in commitment of cells to specific developmental pathways and play an important role in pattern formation (for review, see Scott *et al.,* 1989). In vertebrates, approximately 170 genes which contain homeoboxes have been identified (Stein *et al.,* 1996); among them are the *HOX* genes, which are organized into four clusters in vertebrate genomes (Krumlauf, 1992). Each cluster exhibits similarities to the *Antennapedia* and *Bithorax* complexes of *Drosophila* with regard to the order of gene activation and their spatiotemporal expression pattern. *Hox* genes are expressed in partially overlapping domains, with each expression domain exhibiting a distinct anterior boundary. Thus, cells along the anterior–posterior body axis usually express several *Hox* genes, and it is hypothesized that the developmental fate of cells is encoded by the nature of the *Hox* genes that they express ("*Hox* code": Kessel and Gruss, 1991).

Recently, the NK homeobox family (*NK-1*/*S59, NK-2*/*vnd, NK-3*/*bagpipe,* and *NK-4*/*msh-2*/*tinman,* and *H6*) was

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identified (Kim and Nirenberg, 1989; Stadler *et al.*, 1995). These genes do not map to clusters and show temporal and spatial patterns of expression during development which are distinct from those of the *Hox* genes. *Drosophila msh-2/NK-4/tinman* is expressed in the primitive mesoderm and later, expression becomes restricted to the developing dorsal vessel, the insect equivalent of the vertebrate heart, and visceral mesoderm (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Bodmer *et al.* (1990) have demonstrated that the helix-loop-helix protein *twist* regulates the appearance of *tinman*. Mutations in the *tinman* gene do not affect mesoderm invagination or dorsal spreading, but result in loss of heart formation in the *Drosophila* embryo. In addition, *tinman* is known to regulate *NK-3/bagpipe* expression in the visceral mesoderm (Azpiazu and Frasch, 1993). These observations suggest that *tinman* may be involved in cardiac mesoderm patterning and make it a likely marker for cardiac mesoderm induction.

Several vertebrate *NK-2* homologues have been isolated (for review see Harvey, 1996). There are at least six mouse *Nkx-2* genes which were identified to have significant homology to *Drosophila NK-2* (Guazzi *et al.*, 1990; Price *et al.*, 1992; Lints *et al.*, 1993). Homologs of *Drosophila tinman* have been cloned from the mouse (Lints *et al.*, 1993), chicken, (Schultheiss *et al.*, 1995), *Xenopus* (Tonissen *et al.*, 1994), and zebrafish (Lee *et al.*, 1996). *Nkx-2* factors are DNA-binding proteins which are capable of activating transcription. Nkx2-1, previously identified as thyroid transcription factor-1 (TTF-1), has been implicated in the activation of both thyroid (Civitareale *et al.*, 1989; Javaux *et al.*, 1992) and lung (Bohinski *et al.*, 1994)-specific genes, such as thyroglobulin, thyroperoxidase, and surfactant protein B, in their respective cell types. In *C. elegans*, the NK homolog *CEH-22* has been shown to play a key role in pharyngeal muscle-specific activation of the myosin heavy chain gene, *myo-2* (Okkema and Fire, 1994). Sequence comparison and *in vitro* DNA-binding assays have revealed that *Nkx2-1*, *Nkx2-5*, and *CEH-22* bind to a specific DNA sequence containing TNNAGTG (Guazzi *et al.*, 1990; Bohinski *et al.*, 1994; Okkema and Fire, 1994; Chen and Schwartz, 1995), which is different from the motif 5′-TAAT-3′, recognized by the *Hox* protein family (Damante *et al.*, 1994).

Knockout experiments with *Nkx2-1* (Kimura *et al.*, 1996) and *Nkx2-5* (Lyons *et al.*, 1995) have demonstrated that these genes are required for organogenesis, which suggests that other Nkx-2 family members should have similar roles in development. The mouse *Nkx2-5* knockout revealed that *Nkx2-5* and *tinman* display *different* phenotypes. Dorsal vessel formation was completely blocked in *Drosophila tinman* mutants (Bodmer, 1993). However, *Nkx2-5* knockout mice developed a beating linear heart tube which was defective in the looping process (Lints *et al.*, 1993). This suggests that redundant molecular mechanisms may be responsible for early heart formation, similar to the redundant functions of *Myo D* and *Myf-5* in skeletal muscle formation (Rawls *et al.*, 1995). Chicken and *Xenopus Nkx-2.3* (Buchberger *et al.*, 1996; Evans *et al.*, 1995) are also expressed in the developing heart. However, *cNkx-2.3* is not expressed until after the formation of the linear heart tube (Buchberger *et al.*, 1996); thus, *cNkx-2.3* does not appear to play a redundant role in heart tube formation, which suggests that in the chicken yet a third NK2 family member may be expressed in the early developing heart. For these reasons, we searched for novel NK2 family members that are expressed in the early developing heart. We have isolated a new NK2-related chicken gene, *cNkx-2.8*, and compared the deduced cNkx-2.8 protein sequences with previously published NK2 homeobox proteins. We show that *cNkx-2.8* is expressed in the coalescing heart tube, the pericardial coelomic sac, and in a complex pattern in the ventral pharynx. We recently showed that the cardiac α-actin gene was not directly activated by *Nkx2-5* in transfected fibroblasts, but required the collaboration of additional factors, such as serum response factor (SRF; Chen *et al.*, 1996; Chen and Schwartz, 1996). Here, we also demonstrate that cNkx-2.8 can transactivate a minimal promoter and that cNkx-2.8 and SRF can synergistically activate the cardiac α-actin promoter in a noncardiac muscle cell line. These transactivation data suggest that Nkx2-5 and cNkx-2.8 in combination with SRF may play a role in early heart formation and may act in a combinatorial fashion. This combinatorial mechanism of action, the existence of multiple NK-2 family members, and their partially overlapping patterns of expression are discussed within the framework of a ''Nkx code.''

MATERIALS AND METHODS

cNkx-2.8 cDNA Isolation and Sequencing

To isolate novel Nkx cDNAs, 1.2×10^6 recombinants from a HH stage 15–16 chicken heart Lambda ZAPII cDNA library (Stratagene, La Jolla, CA) were screened under low stringency (55°C; Church and Gilbert, 1984) with a radiolabeled murine *Nkx2-5* cDNA probe encoding the homeobox and the NK2-specific domain (Lints *et al.*, 1993). Of eight primary positives, seven were plaque purified and excised with helper phage as Bluescript II (SK^-) plasmids. Partial sequencing of each clone revealed that one clone contained a poly(A) tail and an open reading frame which encoded for a third helix of a homeobox and an NK-2-specific domain (Fig. 1A). This clone was sequenced in its entirety in both directions using standard dideoxy methodology.

Repeated screening of the heart cDNA library with the radiolabeled partial cDNA revealed no further clones. Thus, the remainder of the cDNA sequence was isolated using a combination of genomic DNA screening and 5′RACE. Recombinant (10^6) clones from a chicken genomic library (Clontech, Palo Alto, CA) were screened with a 134-bp PCR-generated [α-^{32}P]dCTP-labeled probe (forward primer 5′-AAAAATCTGGTTCCAGAACCG-3′, reverse primer 5′-AAAGCAAGGCTTGCCATC-3′; Fig. 1A). Of the six primary clones that were plaque purified, two clones were homologous to our cDNA, as determined by PCR analysis with the above primers. In addition, the homeodomain was amplified using the following degenerate oligonucleotides: 5′-GARYTNGARMGNMGNTT-3′ (homeodomain codons ELERRF, sense) and 5′-AACCADATYTTN-ACYTGNGT-3′ (homeodomain codons FWIKVQT, antisense). A 5.4-kbp *Xho*I fragment was subcloned into pBluescript (KS^-) and a 1986-bp *Xho*I/*Hin*dIII fragment was sequenced.

5′ Rapid Amplification of cDNA Ends (5′ RACE) to Obtain the 5′ Sequence of Nkx-2.8

First-strand cDNA synthesis was performed with 5 μg of total RNA and random primers. The reaction was performed in a 40-μl vol containing 1 m*M* dithiothreitol, 50 m*M* Tris–HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 1 m*M* each dNTP, 100 ng random primer, 20 U of RNAguard ribonuclease inhibitor (Pharmacia Biotech; Piscataway, NJ), and 50 U of MMLV reverse transcriptase (Stratagene, La Jolla, CA) for 90 min at 37°C. The reverse transcriptase reaction was terminated by incubation at 95°C for 5 min and chilling on ice. RNA was degraded by incubating the samples with RNase A (500 μg/ml) at 37°C for 15 min. Random primers, degraded RNA, enzymes, and salt were removed by QIA-quick PCR purification columns (Qiagen, Chatsworth, CA) and the cDNA was eluted with 35 μl of water. Ligation-anchored PCR was used to extend the partial cDNA in the 5′ direction as described by Ansari-Lari *et al.* (1996). The anchor nucleotide (5′-TTTAGTGAGGGTTAATAA-GCGGCCGCGTCGTGACTGGGGAGVGV-3′) had a 5′ phosphate and was blocked on the 3′ end with an amino group (Geneosys, Houston, TX) so that only the 5′ end of the anchor nucleotide was capable of ligation to the 3′ end of the first-strand cDNA. The anchor nucleotide was ligated to the first-strand cDNA with T4 RNA ligase (Pharmacia Biotech). Samples were purified with by QIA-quick PCR purification columns (Qiagen) and the 5′ RACE template was eluted in 50 μl of water.

A nested PCR strategy was used to isolate the 5′ end of cNkx-2.8 using the following primer: anchor-specific primer (5′-GCG-GCCGCTTATTAACCCTCACTAAA-3′), reverse gene-specific primer 1 (5′-TCCACTCCAACACCTGAGT-3′), and reverse gene-specific primer 2 (5′-GGGAGAACAAAACGCGGGGT-3′). Primary PCR reactions (50 μl, 10 m*M* Tris–HCl (pH 8.3), 75 m*M* KCL, 2 m*M* $MgCl_2$, and 2.5 U *Taq* polymerase) were performed with the anchor-specific primer (40 pmol) and reverse gene-specific primer 1 (40 pmol) and 5 μl anchor-ligated cDNA as template. The amplification sequence consisted of an initial DNA denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min was also performed. The secondary PCR reaction, identical to the primary PCR except that reverse gene-specific primer 2 was used, was performed using 10% of the purified primary PCR product(s) as template. PCR products were visualized on 1.5% agarose gels which were stained with ethidium bromide and subcloned with the TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's recommendations. Agarose gel electrophoresis was performed to determine the size of the subcloned PCR fragments. Southern blot analysis was performed using the *Xho*I/*Hin*dIII genomic fragment as a probe to identify authentic PCR products. Positive subclones were sequenced in their entirety.

RNase Protection, Northern Blot, and RT–PCR Analysis

Total RNA was isolated from HH stage 4 to 16 embryos and Day 4, 5, and 6 chicken embryos (Texas A&M University, College Station, TX) as described by Chomczynski and Sacchi (1987). Day 4, 5, and 6 embryos were sectioned into head, heart, and trunk regions. The head section contained all tissues anterior to the heart, the heart section contain the heart and overlying tissues, while the trunk section contained all tissues posterior to the heart. RNase protection assays were performed according to manufacturer's protocol (Ambion, Austin, TX). The *cNkx-2.8* RNA probe was synthesized with T7 RNA polymerase from a sequencing subclone (bp 308–506) after linearization with *Pvu*II, which adds 237 bp of vector sequence to the RNA probe (Fig. 1A). Poly(A) RNA for Northern blot analysis was isolated from heart and tail total RNA by mRNA separator kit (Clontech) according to the manufacturer's protocol. Poly(A) RNA (10 μg) was size-separated on a 1% agarose/formaldehyde gel, transferred to GeneScreen membrane, and hybridized with the RNA probe described above. First-strand cDNA synthesis was carried out for 1 hr at 42°C in 30 μl of reverse transcriptase buffer (Gibco-BRL) supplemented with 0.5 m*M* each dNTP, 3.3 m*M* dithiothreitol, 4 units of RNase inhibitor, 200 units Superscript reverse transcriptase (Gibco-BRL), and 100 ng random hexamers. Polymerase chain reaction was carried out in a volume of 50 μl, with PCR buffer (Perkin–Elmer) with 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP (Boehringer Mannheim), 40 pmol of each primer, and 2 μl of first-strand cDNA. The following primers were used in the PCR reactions: chicken *Nkx-2.8*—forward primer 5′-ATGCTGCCC ACCCCTTTCTC-3′, reverse primer 5′-TCCAACTCCAACACC-TGAGT-3′; chicken *Nkx-2.5* and *GAPDH* (Schultheiss *et al.*, 1995); chicken *Nkx-2.3*—forward primer 5′-CGATGAT GTTACCGAG-CCCC-3′, reverse primer 5′-GCTTCCTCCGACTCCGCTGC-3′. The amplification sequence consisted of an initial DNA denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C (*cNkx-2.5* and *cNkx-2.8*), 65°C (*Nkx2-3*) or 56°C (*GAPDH*) for 1 min, and extension at 72°C for 1 min. In addition, a final extension at 72°C for 5 min was performed.

Whole-Mount and Section in Situ Hybridization

Whole-mounts and section *in situs* were performed as described by Barth and Ivarie (1994) and Albrecht *et al.* (1997). Digoxigenin-labeled RNA probes were synthesized from the partial cDNA covering nucleotides 308–1018 by cutting with *Eco*RI and transcribing with T7 RNA polymerase (antisense) or cutting with *Xho*I and transcribing with T3 RNA polymerase (sense). Alternatively, *in situs* were performed on 7-μm sections. Sections were hybridized overnight at 58°C and washed at 64°C. Sections were processed for emulsion autoradiography, poststained with Hoechst 33258, and visualized by epifluorescence and dark-field microscopy. Sense probes in all cases showed background levels of hybridization in all tissues (data not shown).

Recombinant Plasmids

Full-length coding cDNA was generated by Expand high-fidelity PCR system (Boehringer Mannheim, Indianapolis, IN) with the following primers: forward (5′-GCTCTAGAATGCTGCCCACCC-CTTTCTC-3′) and reverse (5′-GCGGATCCTGGGCCACATCTC-AACTGG-3′). The PCR product was subcloned with the TA cloning kit (Invitrogen) and sequenced. The chicken *Nkx-2.8* expression plasmid pCGN-cNKx-2.8 was constructed by subcloning the PCR product into the pCGN vector (Tanka and Herr, 1990).

Transfection Assays in CV-1 Cultures

Proliferating CV-1 fibroblasts were transfected with a total of 1.8 μg of DNA, composed of 3 × (A20)-TATA-LUC (1 μg; Chen and Schwartz, 1995) in the presence of pCGN-cNkx-2.8 and/or pCGN-SRF (150 ng, gift of Dr. Ron Prywes) and balanced with the parental expression vector, complexed with lipofectamine (Gibco). Cells were harvested 48 hr later and assayed for reporter gene activity and protein content as described previously (Chen and Schwartz,

1995). Data are expressed as normalized luciferase activity relative to baseline reporter gene activity. All experiments were repeated to ensure reproducibility.

Electrophoretic Mobility Shift Assays (EMSA)

Whole cell extracts were prepared form CV-1 cells 72 hr following transfection with pCGN-Nkx-2.8 or pCGN expression plasmids as described by (Lassar *et al.*, 1989). Double-stranded oligonucleotides corresponding to A20 oligo (Chen and Schwartz, 1995), avian cardiac α-actin SRE1 (Chen and Schwartz, 1996), Egr-1 (Cao *et al.*, 1990), and the consensus Nkx2-1-binding site (Chen and Schwartz, 1996) were synthesized and used in EMSAs. EMSA were performed in a 20-μl reaction volume at room temperature. Each reaction contained 1 μg of double-stranded poly(dI-dC) 0.0025 pmol end-labeled oligo (50,000 cpm) and 10 μg of whole cell extract in 10 m*M* Tris, pH 8.0, 1 m*M* dithiothreitol, 1 m*M* sodium phosphate, and 75 m*M* NaCl. Reactions were incubated in the absence of probe for 10 min, the probe was then added, and the reactions were incubated for an additional 10 min. Protein–DNA complexes were size separated on a 0.5× TBE 5% polyacrylamide gel for 4 hr at 100 V.

RESULTS

Cloning of cNkx-2.8

A novel member of the Nkx-2 family was isolated by a combination of low-stringency cDNA library screening, genomic library screening, and 5′ RACE. A HH stage 15/16 chicken heart cDNA library was screened under low-stringency conditions with a subfragment of the mouse *Nkx2-5* cDNA which encodes for the homeobox and the NK2-specific domain. Among the clones isolated, we found a partial cDNA of 710 bp (Fig. 1A) with an open reading frame of 93 amino acids which overlapped the third helix of a homeobox and the proline–valine-rich NK-2-specific domain, and comparison of the coding sequences indicated a few amino acid differences from cNkx-2.5 (Schultheiss *et al.*, 1995) and cNkx-2.3 (Buchberger *et al.*, 1996). A chicken genomic library was then screened with a portion of the partial cDNA (Fig. 1A), and six genomic clones were isolated. Two of these genomic clones contained sequences identical to our cDNA clone. A 5.4-kb *Xho*I genomic DNA fragment was subcloned and a 1986-bp *Xho*I/*Hin*dIII fragment was sequenced. From this sequence, we were able to determine the homeodomain sequence (Figs. 1B and 2A). However, no amino terminal *tinman* (TN) domain, which is present in all previously identified vertebrate NK-2 homologs, was identified in any open reading frame 5′ of the homeobox sequence. We then used ligation-anchored 5′-RACE to find the start methionine of our cDNA. Three 5′ RACE cDNA extensions were isolated which encoded for a TN domain. All three 5′ RACE extensions terminated in a C-rich region just 5′ of the start methionine (Figs. 1A and 1B).

Comparison of the homeodomain amino acid sequence with other NK family members revealed a 92% amino acid sequence identity to *Xenopus*, chicken, and zebrafish Nkx2-5 homeodomains (Fig. 2A). The homeodomains of all previously isolated NK family members have a proline residue at position 29, except *msh-2*, which has an alanine residue, whereas cNkx-2.8 has a leucine. In addition, *cNkx-2.8* encoded for a threonine at position 11 and a glutamine at position 39, which are only found in zebrafish nkx-2.7 and mouse Nkx2-6, respectively. In addition to the homeodomain, all NK-2 family members have two other highly conserved amino acid sequences: (1) the amino terminal TN domain and (2) the NK-2-specific domain (Harvey, 1996). The cNkx-2.8 NK-2-specific domain is identical to all *Nkx2-3* and *Nkx2-2* clones isolated to date (Fig. 2B) and the TN domain is also highly conserved (Fig. 2B). Furthermore, *cNkx-2.8* encodes for the shortest Nkx-2 homeodomain protein isolated to date. Of note, the carboxy terminus of cNkx-2.8 does not share identifiable sequence homology with either cNkx-2.5 or cNkx-2.3, which have a conserved GIRAW amino acid sequence (Buchberger *et al.*, 1996). From these results we conclude that cNkx-2.8 is a novel NK-2 family member.

cNkx-2.8 Expression Pattern

A *cNkx-2.8* RNA probe made from the partial cDNA (bp 308–506; Fig. 1A) was radiolabeled and used in RNase protection assays to determine the expression pattern of *cNkx-2.8*. *cNkx-2.8* mRNA was detected in the heart and trunk regions of Embryonic Day 4 and 5 chicken embryos and the tail region of Day 6 chicken embryos (Fig. 3A). Northern blot analysis was performed with 10 μg of poly(A) mRNA isolated from the heart and trunk and from heart regions of Day 4 chicken embryos to determine the size of *cNkx-2.8* transcripts. *cNkx-2.8* appears to encode for a transcript of 1.1 to 1.5 kb (Fig. 3B). The broad band of *cNkx-2.8* detected on the Northern blot suggests the presence of multiple-sized transcripts, although no distinct bands were detectable upon shorter exposure of the autoradiographs. The well-defined bands for GAPDH and β-actin demonstrate that RNA degradation had not occurred for *cNkx-2.8* RNA transcripts.

In order to compare the temporal expression patterns of the chicken Nkx-2 family members, the earliest developmental stage at which *cNkx-2.3*, *cNkx-2.5*, and *cNkx-2.8* were expressed was determined by RT–PCR analysis (Fig. 3C). Total RNA was isolated from HH-staged whole chicken embryos. *cNkx-2.3* and *cNkx-2.5* transcripts were first detected at HH stage 5. Schultheiss *et al.* (1995) demonstrated by whole-mount analysis that *cNkx-2.5* was first expressed at HH stage 5, where it formed a crescent which extended from the lateral plate at the level of Hensen's node to a point anterior to the tip of the head process. However, Buchberger *et al.* (1996) did not observe the presence of cNkx-2.3 transcripts in the developing embryo until stage 16 of development, where expression was localized to the dorsolateral aspect of the neural tube. In contrast, *cNkx-2.8* transcripts were detected at all HH stages tested, including HH stage 4. The presence of *cNkx-2.8* PCR product cannot be due to genomic contamination because the PCR primers were designed so that they span an intron (data not shown). Similarly, if the intron placement in *cNkx-2.3* and *cNkx-2.5* is similar to *cNkx-2.8*, then the presence of their PCR

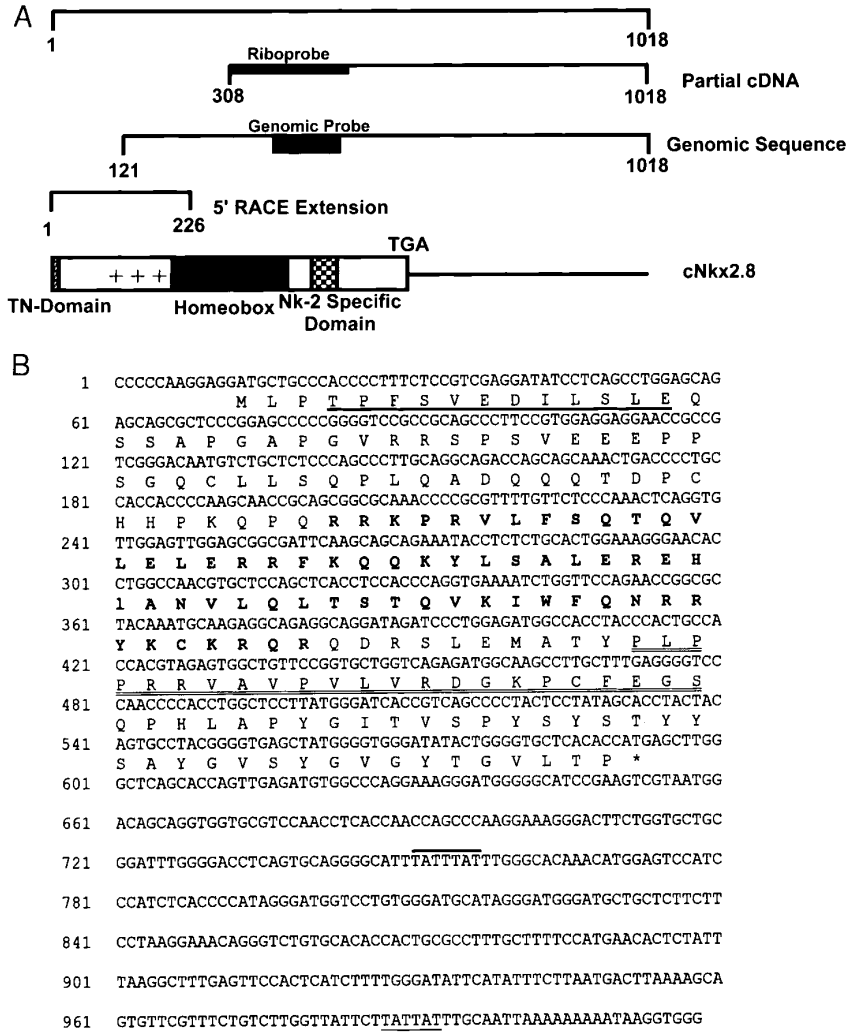

FIG. 1. The structure and deduced amino acid sequence of the chicken Nkx-2.8 cDNA encoded by the λA-5 clone, genomic sequence, and 5′ RACE extension. (A) A schematic representation of the structure of the λA-5 partial cDNA, genomic sequence, and 5′ RACE extension. The sequences which are encoded by each source are indicated below the representations. The sequences used for RNase protection and genomic screening are indicated as black boxes below the λA-5 partial cDNA clone and genomic sequence, respectively. The homeobox is shown as a black box, the NK-2-specific domain as a checkerboard box, the TN domain as a dotted box, an acidic domain as +, and the 3′ UTR as a line. (B) The nucleotide sequence and amino acid sequence of the chicken Nkx-2.8 protein. A mRNA destabilization sequence in the 3′ UTR is overlined and a polyadenylation sequence is underlined. The amino acid sequence is shown below the nucleotide sequence. The TN domain is underlined, the homeodomain is shown in bold type, and the NK-2-specific domain is double-underlined.

products could not be due to genomic contamination. In addition, the PCR reactions lacking reverse transcriptase did not produce a band (data not shown). Thus, *cNkx-2.8* may be the first Nkx-2 family member expressed in the developing embryo.

In Situ Analysis of cNkx-2.8 Expression

A series of section and whole-mount *in situ* hybridization experiments was performed on HH-staged chicken embryos with *cNkx-2.8* specific RNA probes to determine the temporal and spatial expression patterns of cNkx-2.8. In transverse sections, weak cNkx-2.8 expression was first detected in stage 7 embryos, where it was localized to the splanchnopleura (Fig. 4A). By stage 8, *cNkx-2.8* expression in the promyocardium was significantly stronger (Fig. 4B) and was localized to the anterior margin of the anterior intestinal portal (Fig. 4G). In contrast to *cNkx-2.5* (Schultheiss *et al.*, 1995), *cNkx-2.8* was not expressed in the ectodermal tissue overlying the splanchnopleura expression pattern of cNkx-2.8 (Figs. 4A and 4B). By stage 9, *cNkx-2.8* was expressed in the coalescing heart tube and in the ventral pharyngeal endoderm (Fig. 4C). By stage 10, strong *cNkx-2.8* expression was detected in the linear heart tube (Figs. 4D and 4E), the

A

Organism	Gene Name	1 10 20 30 40 50 60	Percent Homology
Chicken	cNkx-2.8	RRKPRVLFSQTQVLELERRFKQQKYLSALEREHLANVLQLTSTQVKIWFQNRRYKCKRQR	100
Frog	XNkx-2.5	----------A--Y----------P--D------K----------	92
Chicken	cNkx-2.5	----------A--Y----------P--D------K----------	92
Fish	nkx2.5	----------A--Y----------P--D------K----------	92
Mouse	Nkx2-3	----------A--F---------R----P-------S-K----------	90
Chicken	cNkx-2.3:	----------A--F---------R----P-------S-K----------	90
Frog	XNkx-2.3:	----------A--F---------R----P-------S-K----------	90
Fish	nkx2.3:	----------A--F---------R----P------ST-K----------	88
Fish	nkx2.7:	----------F---------R----P--D---LA-K----------	88
Human	CSX:	----------A--Y---------R----P--DQ--S--K----------	87
Mouse	Nkx2-5:	----------A--Y---------R----P--DQ--S--K----------	87
Mouse	NKx2-4:	R------A--A---------R----P------SS-K----------M---A	84
Mouse	Nkx2-6:	Q--S------A---A--------R--T-P------SA----------S-S--	82
Canine	TTF-1:	---R------A--Y----------P------SMIH--P----------M---A	82
Human	TTF-1:	---R------A--Y----------P------SMIH--P----------M---A	82
Rat	TTF-1:	---R------A--Y----------P------SMIH--P----------M---A	82
Flatworm	Dth-2:	---R-I----A-IY----------P-------LIN--P---------H------SQ	78
Mouse	Nkx-2.2:	K--R-----KA-TY------R--R----P------SLIR--P---------H---M--A-	72
Leech	Lox-10:	---R-I----A-IY------R-------P------TFIG--P---------H---T-KSK	72
Fish	nk2.2:	K--R-----KA-TY------R--R----P------SILR--P---------H---M--A-	72
Flatworm	Dth-1:	K--R-----KK-I-----H-R-K-----P-------LIG-SP---------H---M--AH	70
Fruitfly	NK2/vnd:	K--R----TKA-TY------R--R----P------SLIR--P---------H---T--AQ	68
Fruitfly	msh-2:	K---------A------C--RLK---TGA---QK-N-SA----------S--GD	68
Frog	XeNK2:	K--R----SKA-TY------R--R----P------SLIR--P---------H---T--AQ	68
Flatworm	ceh-22:	K--R----TKA-TY------RS------P---A--MQIR--P---------H---T-KSH	65
Fruitfly	bagpipe:	KKRS-AA--HA--F-----FA--R---GP--SEM-KS-R--E----------T--KQ	60

Helix I Helix II Helix III

B

Gene	TN Domain	NK2-Specific Domain
cNkx-2.8:	TPFSVEDILSLE	PLPPRR**VAVPVLV**RDGKPCFEGS
nkx2.7:	-----K---K--	LAG----------HGAP
Nkx2-6:		--A----------L-----LDP-
XNkx-2.5:	-----K---N--	LP----I----------LGE-
cNkx-2.5:	-----K---N--	IP----I----------LGE-
nkx2.5:	-----R---N--	IA----IS----------LGDT
CSX:	-----K---N--	-P----I----------LGD-
Nkx2-5:	-----K---N--	-P----I----------LGDP
Nkx2-4		S----------K-----QN-A
cNkx-2.3:	--S--K------	-RA----------LG--
XNkx-2.3:	-----K---N--	-P----------IG--
Nkx2-3:	-----K---N--	-P----------VTP-
nkx2.3:	-----K---K--	-P----------LT--
nk2.2:	-G---K---D-P	LPS----------HTLK
Nkx2-2:		LPS----------HALK
Nkx2-1:	-----S----PL	LPS----------K-----QA-A
vnd:	SG-HIS---N--	LPS----------K-----LGD-
XeNK2:	-G---K---D-P	LPS----------HTKA
msh-2:	-----K---NMV	
bagpipe:	----IN---TRS	LGASK--PIQ------STTYAHM
Xbap:	----IQA--NRK	APAAKK---K-----QRQYHP-E
eh 1:	L---IDN----D	

ventral pharyngeal endoderm dorsal to the heart tube (Figs. 4D and E), and the dorsal half of the vitelline veins (Fig. 4F). In addition, whole-mount analysis demonstrated that *cNkx-2.8* was expressed in the vitelline veins (Figs. 4H and 4I). *cNKx-2.8* expression is more pronounced on the left side of the embryo (Figs. 4E and 4H). However, *cNkx-2.8* was not expressed in the endocardium (Figs. 4D, 4E, and 4F) or the endoderm dorsal to the vitelline veins (Fig. 4F). There was a dramatic increase in the level of *cNkx-2.8* expression in the linear heart tube between the 9 and 11 somite stages (compare Figs. 4H and 4I). In addition, *cNkx-2.8* was expressed transitorily in the telencephalon. The early expression pattern of cNkx-2.8 was similar to that of cNkx-2.5 (Schultheiss *et al.*, 1995), except that unlike *cNkx-2.5*, which was first expressed at stage 5, *cNkx-2.8* expression was first observed by section *in situ* at stage 7 and was not detected in the ectoderm overlying the splanchnopleura expression pattern. Note, however that RT–PCR analysis detects *cNkx-2.8* expression prior to that of *cNkx-2.5* expression. Possibly early expression of Nkx-2.8 is diffuse and not localized. Early expression in heart primordia indicates that like cNkx-2.5, cNkx-2.8 may play an important role in early heart development.

Transverse sections through the cardiac region of a stage 13 embryo demonstrated that *cNkx-2.8* was expressed primarily in the ventral pharyngeal endoderm dorsal to the looping heart (Fig. 5A), with undetectable levels of expression in the medial portion of the ventral pharyngeal endoderm. In addition, very low levels of *cNkx-2.8* expression were detected in the intermediate mesoderm. In contrast to *cNkx-2.5* (Schultheiss *et al.*, 1995), which by stage 14 was expressed primarily in the looping heart tube, stage 13 whole mounts revealed that cNkx-2.8 was not expressed in the looping heart tube or the anterior intestinal portal but was only expressed in a symmetrical pattern in the tissues overlying the looping heart, with the highest level of expression located at the base of the second pharyngeal cleft (Figs. 5D and 5E). Similarly at stage 15, *cNkx-2.8* was primarily expressed in the ventral pharyngeal endoderm dorsal to the looping heart, the ectoderm lateral to the aortic sac, and the aortic sac (Fig. 5B). *cNkx-2.8* was also expressed in the mesenchyme and the epithelium of the pericardial coelomic sac dorsal to the heart tube (Fig. 5C). In addition, stage 15 whole-mounts demonstrate the overall expression pattern of *cNkx-2.8* which was expressed in the outflow tract and tissues underlying the looping heart tube (Fig. 5F). Thus, the *cNkx-2.8* gene has a temporally and spatially restricted pattern of expression which is distinct from that of *cNkx-2.5*.

By stage 17, *cNkx-2.8* was expressed in the lateral portion of the ventral pharyngeal endoderm, the surface ectoderm lateral to the intermediate mesoderm, and the sinus venosus, similar to stage 15 (compare Figs. 5B and 6A). However, *cNkx-2.8* expression in the pharyngeal arch region was limited primarily to the second and third pharyngeal pouches, with a low level of expression detected in the first pharyngeal pouch in a region adjacent to the second pharyngeal arch (Fig. 6D). Removal of the head and looping heart of the embryo pictured in Fig. 6D revealed that *cNkx-2.8* was expressed in the sinuous venosus, the caudal half of the aortic sac, and in a symmetrical pattern in the second and third pharyngeal pouches (Fig. 6E). When the embryo in Fig. 6E was sectioned down the midline, it revealed that *cNkx-2.8* was expressed in the ventral portions of the second and third pharyngeal pouches, the sinuous venosus, the aortic sac, and the ventral pharyngeal endoderm from pharyngeal arch 1 to a point posterior to the sinus venosus (Fig. 6F). By stage 20, *cNkx-2.8* was expressed in the endoderm of the second and third pharyngeal pouches (Fig. 6B). In addition, *cNkx-2.8* was expressed in the aortic sac and the epithelial lining and mesenchymal tissues of the pericardial coelomic sac dorsal to the heart (Figs. 6B and 6C). However, *cNkx-2.8* was not expressed in the developing lung buds or the liver rudiment.

cNkx-2.8 Binds to Consensus Nkx2-5 and Nkx2-1 DNA-Binding Sites

Nkx2-5 and Nkx2-1 bind to similar DNA sequences (Damante and Di Lauro, 1991; Chen and Schwartz, 1995) and are both capable of transactivating the lung-specific CC10 promoter (Ray *et al.*, 1996), which suggests that NK-2 family members may play similar roles in different tissues. The third helix of the homeodomain, which is believed to determine DNA-binding specificity, of cNkx-2.8 is identical to that of Nkx2-5 and very similar to Nkx2-1, which suggests that cNkx-2.8 should bind to similar DNA sequences. Therefore, to determine if cNkx-2.8 binds to known Nkx2-5 and Nkx2-1 DNA-binding sites, EMSAs were performed

FIG. 2. Comparison of the homeodomain, TN domain, and NK-2-specific amino acid sequences with other known NK family members. (A) The homeodomain amino acid sequence is represented along with the gene name and species of origin. All sequences are compared to cNkx-2.8 and identical amino acids are indicated by a dash. The leucine at position 29 (bold type) is unique to cNkx-2.8. The predicted homeodomain helixes are indicated at the bottom of the figure. The percentage amino acid identity of each homeodomain to cNkx-2.8 is indicated on the right. (B) Comparison of the TN domain and NK-2-specific domains. Missing amino acid sequences either do not exist or are not known. The eh 1 sequence was recently identified as an inhibitory domain present in several homeobox containing proteins (Smith and Jaynes, 1996). Reference for individual genes are as follows: *XNkx-2.5* (Tonissen *et al.*, 1994); *cNkx-2.5* (Schultheiss *et al.*, 1995); *nkx-2.3*/*nkx-2.5*/*nkx-2.7* (Lee *et al.*, 1996); *Nkx2-1*/*Nkx2-2*/*Nkx2-3*/*Nkx2-4* (Price *et al.*, 1992); *cNkx-2.3* (Buchberger *et al.*, 1996); *XNkx-2.3* (Evans *et al.*, 1995); *CSX* (Komuro and Izumo, 1993); *Nkx-2.3*/*Nkx-2.5*/*Nkx-2.6* (Lints *et al.*, 1993); canine, human, and rat *TTF-1* (Mizuno *et al.*, 1991; Saiardi *et al.*, 1995; Van Renterghem *et al.*, 1995); *Lox10* (Nardelli-Haefliger and Shankland, 1993); *nk2.2* (Barth and Wilson, 1995); *vnd* (Jimenez *et al.*, 1995); *msh-2* (Bodmer *et al.*, 1990); *bagpipe* (Azpiazu and Frasch, 1993); *ceh-22* (Okkema and Fire, 1994); *XeNK2* (Saha *et al.*, 1993); *Dth-1*/*Dth-2* (Garcia-Fernandez *et al.*, 1991).

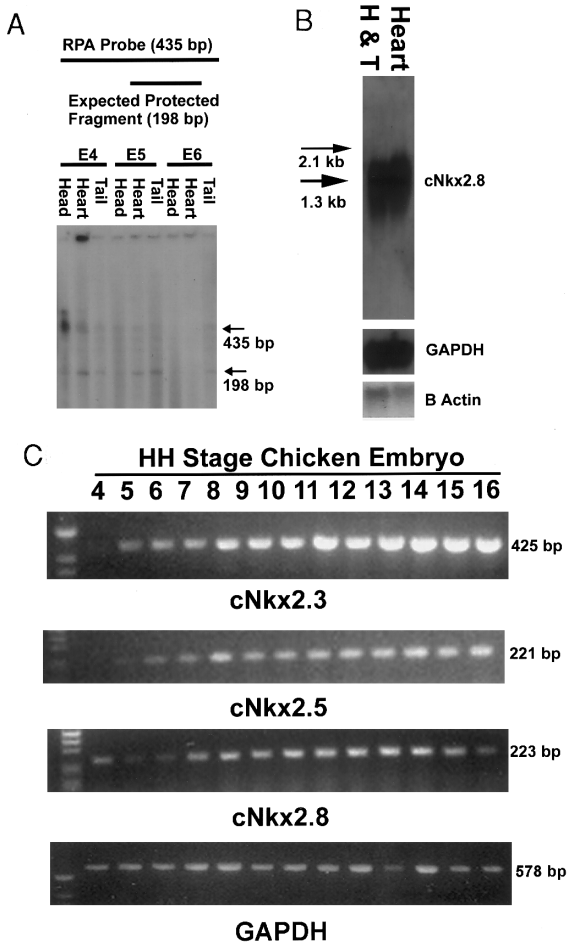

FIG. 3. RNase protection, Northern blot, and RT–PCR analyses of *cNkx-2.8* expression in the developing chicken embryo. (A) Total RNA was isolated from Day 4, 5, and 6 chicken embryos sectioned into head, heart, and trunk regions. Thirty micrograms of total RNA was used for each RNase protection assay. The size of the RNA probe (435 bp) and expected size of the protected fragment (198 bp) are depicted above the RNase protection blot and their placements on the RNase protection blot are indicated with arrows. (B) Northern blot analysis of *cNkx-2.8.* Thirty micrograms of poly(A) mRNA was isolated from Day 4 chicken embryos, size separated on a formaldehyde–agarose gel, and transferred to GeneScreen. The Northern blot was probed with *cNkx-2.8,* chicken *β-actin,* and chicken *GAPDH* radiolabeled probes. The size of the *β-actin* and *GAPDH* transcripts are indicated by small and large arrows, respectively. (C) RT–PCR analysis of *cNkx-2.3, cNkx-2.5, cNkx-2.8,* and *GAPDH* expression in HH-staged chicken embryos. Total RNA (5 μg) isolated from HH-staged chicken embryos was reverse transcribed and 1/10 of this mixture was PCR amplified with indicated PCR primers. Following PCR amplification, PCR products were size separated by electrophoresis through a 1.5% agarose gel.

with whole cell extracts prepared from CV-1 cells transiently transfected with the *cNkx-2.8* expression plasmid, *pCGN-cNkx-2.8,* or the parent plasmid, *pCGN,* and the double-stranded radiolabeled A20 oligonucleotide (Fig. 7A). The radiolabeled A20 oligonucleotide probe bound a single protein complex which was abolished by the addition of unlabeled A20 or by unlabeled Nkx2-1 consensus DNA-binding site oligonucleotide. However, the cardiac α-actin *SRE1* and SRF *Egr-1* oligonucleotides were unable to compete for cNkx-2.8 protein binding. These data demonstrate that cNkx-2.8 can bind specifically to known Nkx-2 DNA-binding sites.

cNkx-2.8 Acts as a Transcriptional Activator

The ability of cNkx-2.8 to transactivate a minimal promoter was investigated to assess the capability of cNkx-2.8 to function as a transcription factor. Previously, this lab generated two reporter constructs which contain a minimal cardiac α-actin *tata* promoter with and without a multimerized Nkx2-5 DNA-binding site (Chen and Schwartz, 1995), A20 tata luciferase and -58 luciferase, respectively. CV-1 cells were transiently cotransfected with varying amounts of pCGN-cNkx-2.8 and these reporter constructs. The negative control plasmid (pCGN) was unable to transactivate either reporter construct (Fig. 7B). In contrast, the forced expression of cNkx-2.8 transactivated the A20 tata luciferase reporter construct in a dosewise manner up to 16-fold (Fig. 7B).

To determine if the increase in transcriptional activity was dependent upon binding of cNkx-2.8 to the A20 motif, the cNkx-2.8 expression plasmid was transiently cotransfected with the -58 luciferase reporter plasmid, which does not contain the A20 DNA-binding site. Removal of the multimerized A20 DNA-binding site abolished all transcriptional activity (Fig. 7B), demonstrating that the transcriptional activation of A20 tata luciferase reporter construct was dependent upon an intact A20 DNA-binding site.

Previously, it has been demonstrated that murine Nkx2-5 and SRF could coactivate a minimal cardiac α-actin promoter in 10T1/2 mouse fibroblasts (Chen and Schwartz, 1996). To test whether cNkx-2.8 and SRF could transactivate transcription of a minimal SRE containing cardiac α-actin promoter (-100 luciferase), CV-1 cells were transiently cotransfected with cNkx-2.8 and SRF. SRF alone moderately increased reporter activity (twofold); whereas SRF and cNkx-2.8 synergistically transactivated the reporter construct (sixfold). In contrast, cNkx-2.8 alone was unable to transactivate the reporter construct (Fig. 7C) at any concentrations tested (data not shown). This inability of cNkx-2.8 to transactivate the minimal SRE containing cardiac α-actin promoter was not unexpected because the cardiac α-actin SRE1 was unable to compete for cNkx-2.8 binding in EMSAs (Fig. 7A). Taken together, these data indicate that cNkx-2.8 can act as a transcriptional activator similar to Nkx2-5.

DISCUSSION

Role of cNkx-2.8 in Early Heart Development

Members of the Nkx-2 family of transcription factors play important roles in the regulation of cell lineage differentia-

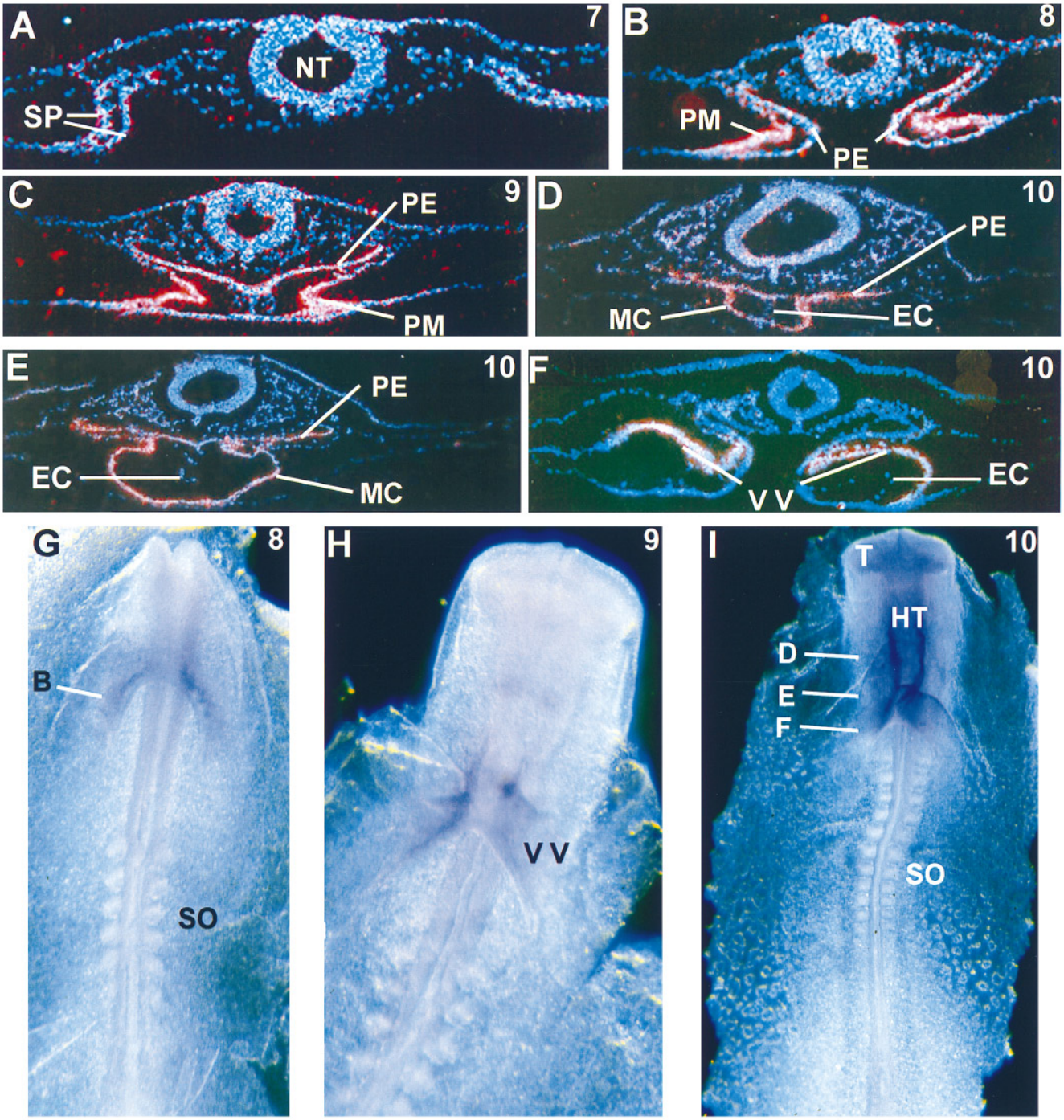

FIG. 4. *In situ* hybridization analysis of *cNkx-2.8* expression on transverse section and whole-mount chicken embryos at HH stages 7–10. The HH stage is indicated in the upper right corner of each picture. (A) Transverse section of a stage 7 chicken embryo. Weak *cNkx-2.8* expression was detected in the splanchnopleura. (B) Transverse section of stage 8, at the level indicated in G. There was a dramatic increase in the level of *cNkx-2.8* expression detected in the promyocardium. (C) Transverse section of stage 9, at the level of the coalescing heart tubes. *cNkx-2.8* expression was detected in the promyocardium but not the endocardium and was also found in the ventral pharyngeal endoderm. (D) Transverse section of a stage 10 chicken embryo, at the level indicated in I. *cNkx-2.8* expression was detected in the myocardium and the ventral pharyngeal endoderm lateral to the aortic sac but not in the endocardium. (E) transverse section of stage 10, at the level indicated in I. *cNkx-2.8* expression was detected in the myocardium and ventral pharyngeal endoderm lateral to the dorsal mesocardium. (F) Transverse section of a stage 10 chicken embryo, at the level indicated in I. *cNkx-2.8* expression was detected in the dorsal half of the vitelline vein but not in the endocardium or the pharyngeal endoderm dorsal to the vitelline vein. (G) Stage 8, ventral view. *cNkx-2.8* expression was detected in the forming heart tubes. (H) Stage 10, 9 somite, ventral view. The predominate signal was found in the coalescing heart tubes. *cNKx2.8* expression is more pronounced on the left side of the embryo. (I) Stage 10, 11 somite, ventral view. There was a dramatic increase in *cNkx-2.8* expression in the linear heart tube and the vitelline veins and a low level of expression in the telencephalon. (EC, endocardium; HT, heart tube; MC, myocardium; NT, neural tube; PE, pharyngeal endoderm; PM, promyocardium; SO, somite; SP, splanchnopleura; T, telencephalon; V V, vitelline vein).

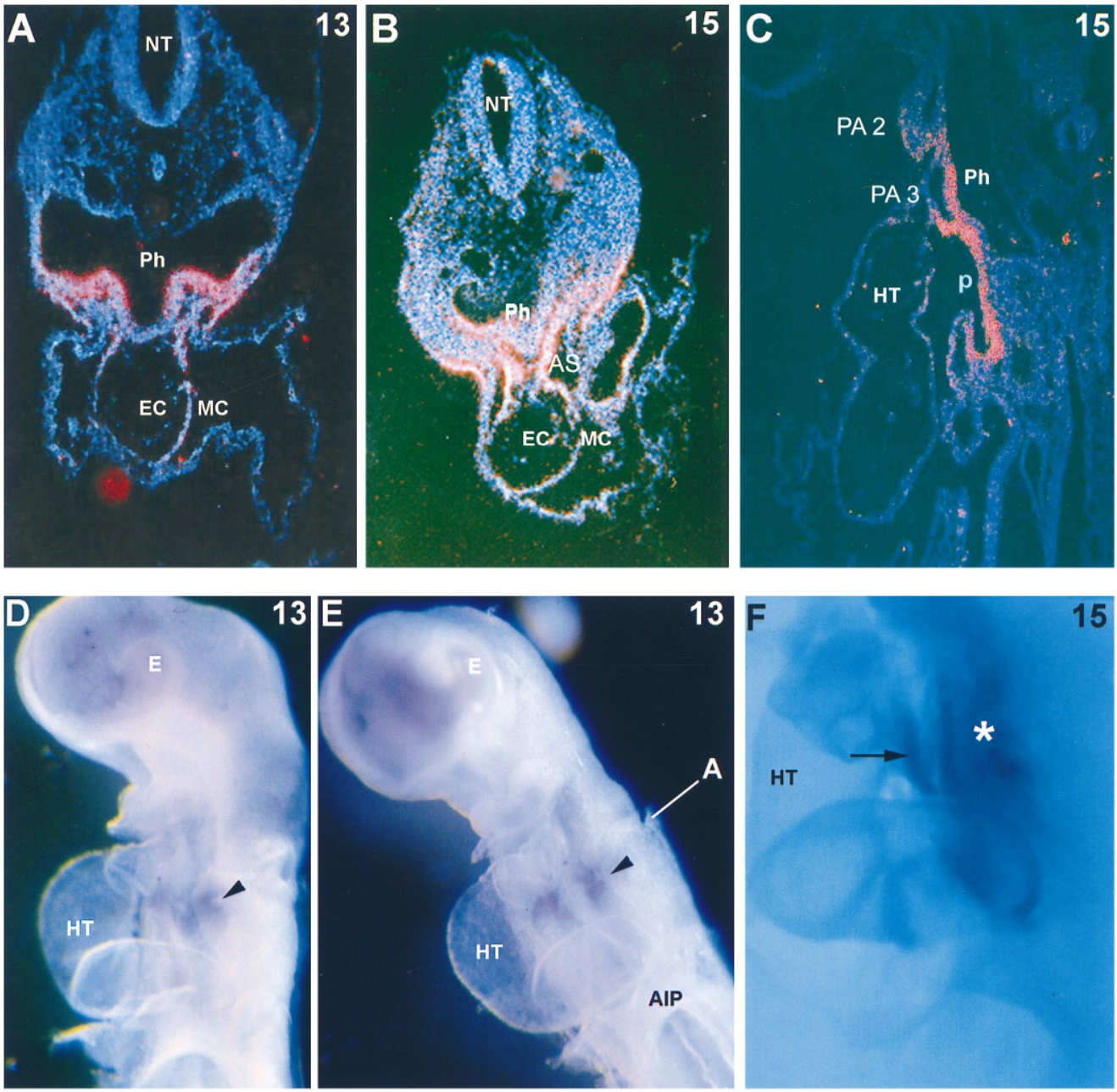

FIG. 5. *In situ* and whole-mount hybridization analyses of *cNkx-2.8* expression in HH stage 13 and 15 chicken embryos. The HH stage is indicated in the upper right of each picture. (A) Transverse section of stage 13, at the level indicated in E. *cNkx-2.8* expression was detected in the lateral but not medial portions of the ventral part of the pharynx. (B) Transverse section of stage 15, *cNkx-2.8* expression was detected in the lateral portion of the ventral pharyngeal endoderm, the aortic sac, and in the ectoderm lateral to the aortic sac. (C) Sagittal section of stage 15, lateral to the midline. *cNkx-2.8* expression was detected in the epithelial tissue of the pericardial sac. (D) Stage 13, side view. *cNkx-2.8* expression was detected in the tissue dorsal to the looping heart tube, but not in the heart tube. (E) Stage 13, ventral view. *cNkx-2.8* was expressed in a symmetrical pattern dorsal to the heart tube with the highest level of expression located at the base of the second pharyngeal cleft (arrowhead). (F) Stage 15, side view. The predominant *cNkx-2.8* signal was found in the tissues dorsal to the looping heart (*) and in the posterior half of the aortic sac (arrow). (AIP, anterior intestinal portal; AS, aortic sac; E, eye; EC, endocardium; HT, heart tube; MC, myocardium; NT, neural tube; P, pericardial coelomic sac; PA, pharyngeal arch; Ph, pharynx).

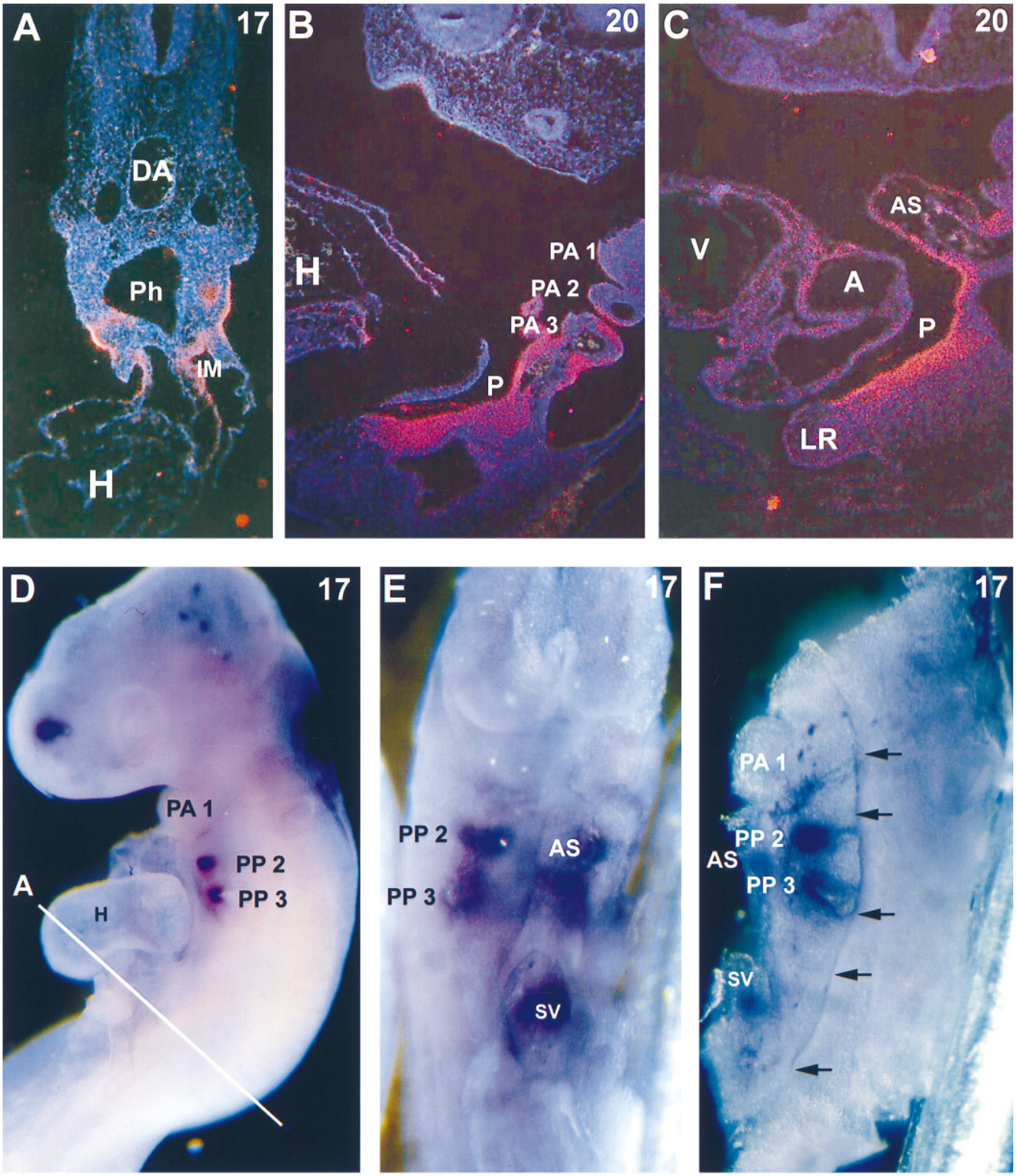

FIG. 6. *In situ* and whole-mount hybridization analyses of *cNkx-2.8* expression in stage 17 and 20 chicken embryos. The HH stage is indicated in the upper right corner of each picture. (A) Transverse section of stage 17, at the level indicated in D. *cNkx-2.8* was expressed in the lateral portion of the ventral pharyngeal endoderm, the intermediate mesoderm, and the ectoderm ventral to the pharynx. (B) A medial sagittal section of stage 20. *cNkx-2.8* expression was detected in the pharyngeal pouch 2 and 3 endoderm and in the epithelium and mesenchyme dorsal to the heart. (C) A lateral sagittal section of stage 20. *cNkx-2.8* was expressed in the aortic sac, the epithelium and mesenchyme dorsal to the heart, but not in the liver rudiment. (D) Stage 17, side view. *cNkx-2.8* expression was detected in the second and third pharyngeal pouches, but not in the heart tube. (E) Stage 17, ventral view of embryo in D with the head and heart tube removed. *cNkx-2.8* was expressed in the sinus venosus, the posterior half of the aortic arch and in a bilateral pattern in the lateral portion of pharyngeal pouches two and three. (F) Stage 17, midline view of embryo in E. *cNkx-2.8* expression was detected in the ventral portion of the second and third pharyngeal pouches, the aortic sac, sinus venosus, and the ventral pharyngeal endoderm from pharyngeal arch two to a point posterior to the sinus venosus (indicated by arrows). (A, atrium; AS, aortic sac; DA, dorsal aorta; IM, intermediate mesoderm; LR, liver rudiment; H, heart; P, pericardial coelomic sac; PA, pharyngeal arch; PP, pharyngeal pouch; SV, sinus venosus; V, ventricle).

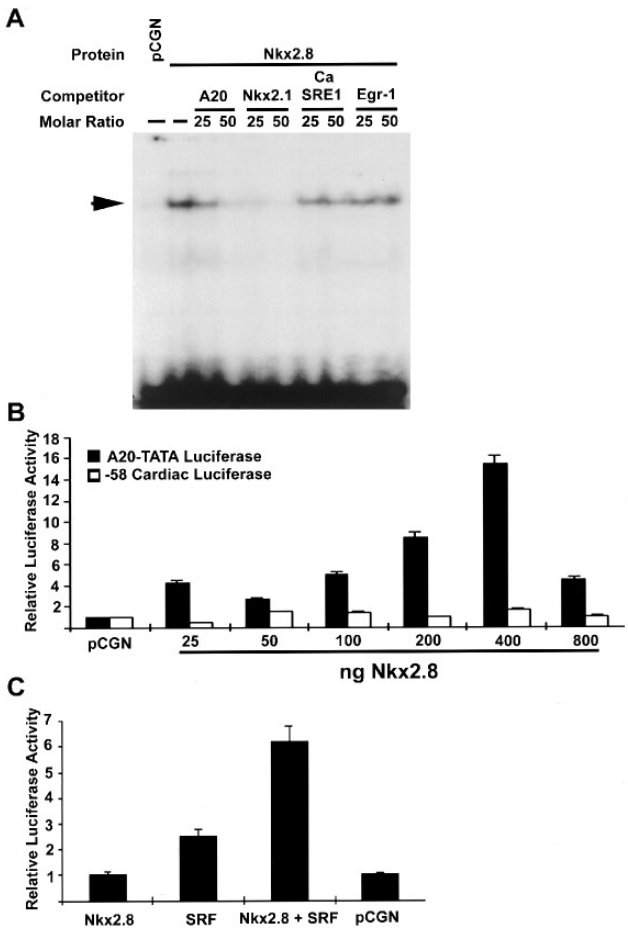

FIG. 7. cNkx-2.8 binding to known *Nkx-2* DNA-binding sites and transactivation of minimal cardiac α-actin promoters in noncardiac muscle cells. (A) The A20 oligonucleotide (Chen and Schwartz, 1995) was used in EMSAs with whole cell extracts prepared from CV-1 cells transiently transfected with *cNkx-2.8* expression plasmid (pCGN-cNkx-2.8) or parent vector (pCGN). Where indicated, binding reactions were preincubated with 25- or 50-fold excess of unlabeled *A20, Nkx2-1, cardiac α-actin SRE1,* or *Egr-1* competitor oligonucleotides. The band which corresponds to cNkx-2.8 nuclear protein complex is indicated with an arrowhead. (B) CV-1 cells were transfected with 0–800 ng of pCGN-cNkx-2.8 and 1 μg of reporter construct (A20 *tata* luciferase or -53 luciferase). Seventy-two hours following transfection, luciferase activity and protein content of cellular extracts were determined. Relative luciferase activity was determined by correcting for differences in protein content and normalizing to the luciferase activity obtained following the cotransfection of either the A20 *tata* luciferase or -58 luciferase with pCGN. The data are presented as relative luciferase activity ± SEM, with the pCGN treatment arbitrarily set to 1. (C) CV-1 cells were cotransfected with 1 μg cardiac α-actin–100 luciferase and 400 ng pCGN-cNkx-2.8 and/or 150 ng pCGN-SRF.

tion during vertebrate development. In this paper, we report the isolation and structural and functional characterization of chicken Nkx-2.8, which is a member of a family of vertebrate homologs to the *Drosophila* gene *tinman*. Structural comparison of the predicted chicken Nkx-2.8 protein with previously characterized Nkx-2 family members revealed a high degree of conservation within the homeobox, NK-2-specific, and TN domains. The homeodomain is closely related to the homeobox sequence of mouse, *Xenopus,* and chicken Nkx2-5 (Lints *et al.,* 1993; Evans *et al.,* 1995; Schultheiss *et al.,* 1995). In addition, Nkx-2.8 protein binds to known Nkx2-5 and Nkx2-1 DNA-binding sites and overexpression of cNkx-2.8 is sufficient to transactivate a minimal cardiac α-actin promoter in noncardiac muscle cells. In contrast to *cNkx-2.3* and *cNkx-2.5,* which are expressed primarily in the embryonic and adult heart, the *cNkx-2.8* gene is expressed in a temporally and spatially restricted pattern during early cardiac development. However, later in embryonic development *cNkx-2.8* is expressed at high levels within the second and third pharyngeal pouches, the aortic sac, and the sinus venosus. These data are consistent with a model wherein cNkx-2.8 regulates a novel molecular program in avian development.

The early expression of *cNkx-2.8* in the lateral plate mesoderm and underlying endoderm is similar to the expression pattern of mouse, chicken, and *Xenopus Nkx2-5* (Lints *et al.,* 1993; Tonissen *et al.,* 1994; Schultheiss *et al.,* 1995). Similarly, the *Drosophila* homolog *tinman* is expressed in cardiogenic mesoderm (Bodmer, 1993). Formation of the heart-like structure, the dorsal vessel, in *Drosophila* is dependent on *tinman* (Bodmer, 1993). However, in *Nkx2-5* null mice, the linear heart tube formed normally, but looping morphogenesis and subsequent developmental steps, such as septation and trabeculation, were impaired (Lyons *et al.,* 1995). These seemingly different functions of *Drosophila tinman* and mouse *Nkx2-5* suggest that *Nkx2-5* is not solely responsible for establishing cardiogenic mesoderm, which may be explained by the presence of additional Nkx-2 genes, which may play redundant roles in early cardiac development. The expression pattern of cNkx-2.8 in early cardiac development and its subsequent lack of expression in the looping heart tube suggests that *cNkx-2.8* may be responsible for the formation of the early heart tube in *Nkx2-5* null mice. Furthermore, the ability of cNkx-2.8 and SRF to cotransactivate a minimal cardiac α-actin promoter in a similar manner to Nkx2-5 (Chen and Schwartz, 1995) also suggests that this may be the case. Thus, Nkx2-5 and cNkx-2.8 may be able to replace each other in early heart development.

Role of cNkx-2.8 in Pharyngeal Endoderm

The pharyngeal arches contain endodermal, ectodermal, and mesenchymal components. The ectodermal and endodermal tissues encase the mesenchyme, which is of paraxial mesoderm and neural crest origin. During development the endoderm of the pharyngeal pouches 1, 2, 3, and 4 gives rise to the auditory tube, the *fossa* of the tonsils, the thymus and inferior parathyroid glands, and the superior parathyroid glands, respectively. The mesenchyme gives rise to muscles, skeletal elements, nerves, and blood vessels which are characteristic of each arch (Le Douarin, 1982; Horstad-

ius, 1950). However, the mechanism of patterning of the pharyngeal arches is poorly understood.

In explant cultures, premigratory neural crest cells do not differentiate into cartilage unless they are cultured with cranial ectoderm or pharyngeal endoderm (Epperlein, 1974; Bee and Thorogood, 1980; Graveson and Armstrong, 1987). Thus, cell–cell interactions must occur between the epithelium and the mesenchyme of the pharyngeal arches for the correct developmental pattern to occur. In addition, ablation of the cardiac neural crest does not affect the number of pharyngeal arches or the segmentation pattern of the pharyngeal apparatus (Bockman *et al.,* 1989). Kirby *et al.* (1997) suggested that this ability of the pharyngeal apparatus to segment in the absence of cardiac neural crest indicates an intrinsic program of the pharyngeal endoderm/ectoderm. However, trunk and mesencephalic neural crest cells cannot substitute for cardiac neural crest cells (Kirby, 1989), which suggests that premigratory neural crest cells have acquired positional patterning prior to pharyngeal arch formation. Thus, it is possible that *cNkx-2.8* may play a role in the signaling cascade between cardiac neural crest cells and the endoderm of the second and third pharyngeal pouches, which is responsible for pharyngeal arch patterning and eventually cardiac neural crest differentiation.

Cardiac neural crest ablation results in outflow tract malformations (Kirby *et al.,* 1985), which include persistent truncus arteriosus, dextroposed aorta, tetralogy of fallot, and double-outlet right ventricle. In addition, cardiac neural crest ablation results in deletion of the thymus and parathyroid, which demonstrates that neural crest cell–endodermal interactions are required for proper pharyngeal arch endoderm differentiation to occur, all of which resembles CATCH-22 and DiGeorge syndrome (DGS). DiGeorge syndrome symptoms include the absence of thymus and parathyroid, abnormal cellular immunity, congenital heart defects, hypocalcemia, and facial dysmorphism (DiGeorge, 1968). Because *cNkx-2.8* was expressed during early heart formation and in the outflow tract, pharyngeal pouch 3, which gives rise to the thymus, it is tempting to speculate that *cNkx-2.8* may play a role in the DiGeorge syndrome phenotype. Similar to *cNkx-2.8,* mouse *Hoxa-3* is expressed in the ventral pharyngeal endoderm, the mesenchyme of the third and fourth pharyngeal arches, and the endoderm of the third and fourth pharyngeal pouches (Gaunt, 1988). Disruption of the *Hoxa-3* gene results in homozygotes that are athymic, aparathyroid, and have reduced thyroid tissue and craniofacial anomalies (Chisaka and Capecchi, 1991). In addition, *pax-1* expression in the endoderm of the third pharyngeal pouch is downregulated, which suggests that the *pax-1* signal is downstream of *Hoxa-3.* However, the downregulation of *pax-1* cannot entirely account for the observed thymic deletion in *Hoxa-3* null mice, because the thymus in *pax-1* null mice is only 30–50% smaller than that in wild-type mice. Manley and Capecchi (1995) suggested that *Hoxa-3* null mice have a defect in pharyngeal pouch endoderm and pharyngeal neural crest mesenchyme and together these two defects cause the severe thymus phenotype. Alternatively, lack of *Hoxa-3* expression in the endoderm may downregulate both *pax-1* and another gene and thus result in the *Hoxa-3* thymus phenotype. It would be interesting to know if *Hoxa-3, pax-1,* and/or neural crest cell–endodermal interactions regulate *cNkx-2.8* gene expression. Notably, it has been suggested that *Hoxb-3* may regulate the expression of thyroid transcription factor-1 (Guazzi *et al.,* 1994), which is a member of the Nkx-2 family of transcription factors.

"Nkx Code"

The partially overlapping expression pattern of *Hox* genes in embryos has lead to the concept of a ''*Hox* code'' (Kessel and Gruss, 1991). The term ''*Hox* code'' means that a particular combination of *Hox* genes is functionally active in a region and thereby specifies the developmental fate of this region. The existence of eight *Nkx-2* family members, their overlapping DNA-binding specificities, and, most importantly, their partially overlapping patterns of expression raises the possibility of a ''Nkx code.'' Figures 8A–8D diagram known *Nkx-2* gene expression patterns in the developing pharyngeal primordium and heart (Lazzaro *et al.,* 1991; Lints *et al.,* 1993; Tonissen *et al.,* 1994; Evans *et al.,* 1995; Schultheiss *et al.,* 1995; Buchberger *et al.,* 1996; Lee *et al.,* 1996). In the pharyngeal region, *Nkx-2.3* and *-2.5* are expressed in broad, partially overlapping domains primarily within ectoderm and endoderm. By contrast, *Nkx2-1* and *-2.8* expression is spatially more restricted; transcripts are found at the site at which the anlagen for thyroid, thymus, and lung reside. It is thus possible that the position and the identities of these organ rudiments are determined by the combinatorial expression of Nkx genes. Whether the genes depicted in Fig. 8A are sufficient to define an unique Nkx code or whether other (e.g., zebrafish nkx-2.7; Lee *et al.,* 1996) and/or yet to be discovered family members are required remains an open question. Of note, numerous *Hox* genes are also expressed in migrating neural crest cells within the pharyngeal arch region (reviewed in Hunt and Krumlauf, 1992) and such *Hox* genes may pattern this part of the embryo, perhaps in conjunction with the *Nkx-2* genes. Several *Hox* genes are expressed in the thyroid as well as in the thymus promordia (Gaunt *et al.,* 1989), indicating a direct involvement in the specification of these tissues.

During early heart formation, *Nkx-2.5, -2.7,* and *-2.8* are expressed in partially overlapping domains in the lateral plate mesoderm and pharyngeal endoderm (Fig. 8B). Similarly, *Xenopus Nkx-2.3* is expressed in the cardiogenic field (Evans *et al.,* 1995). However, *cNkx-2.3* is not expressed in the developing heart until much latter (Buchberger *et al.,* 1996). Buchberger *et al.* (1996) suggested that *cNkx-2.3* and *XNkx2-3* are not analogous genes; however, species–species differences may explain these observed differences. In the linear heart tube, *Nkx-2.5, -2.7,* and *-2.8* are expressed in overlapping domains (Fig. 8B). However, after the linear heart tube has undergone the looping process, *Nkx-2.3, -2.5,* and *-2.8* are expressed in distinct and partially overlapping domains. Whether the expression of an individual or a combination of Nkx genes is sufficient to specify a cardiac cell

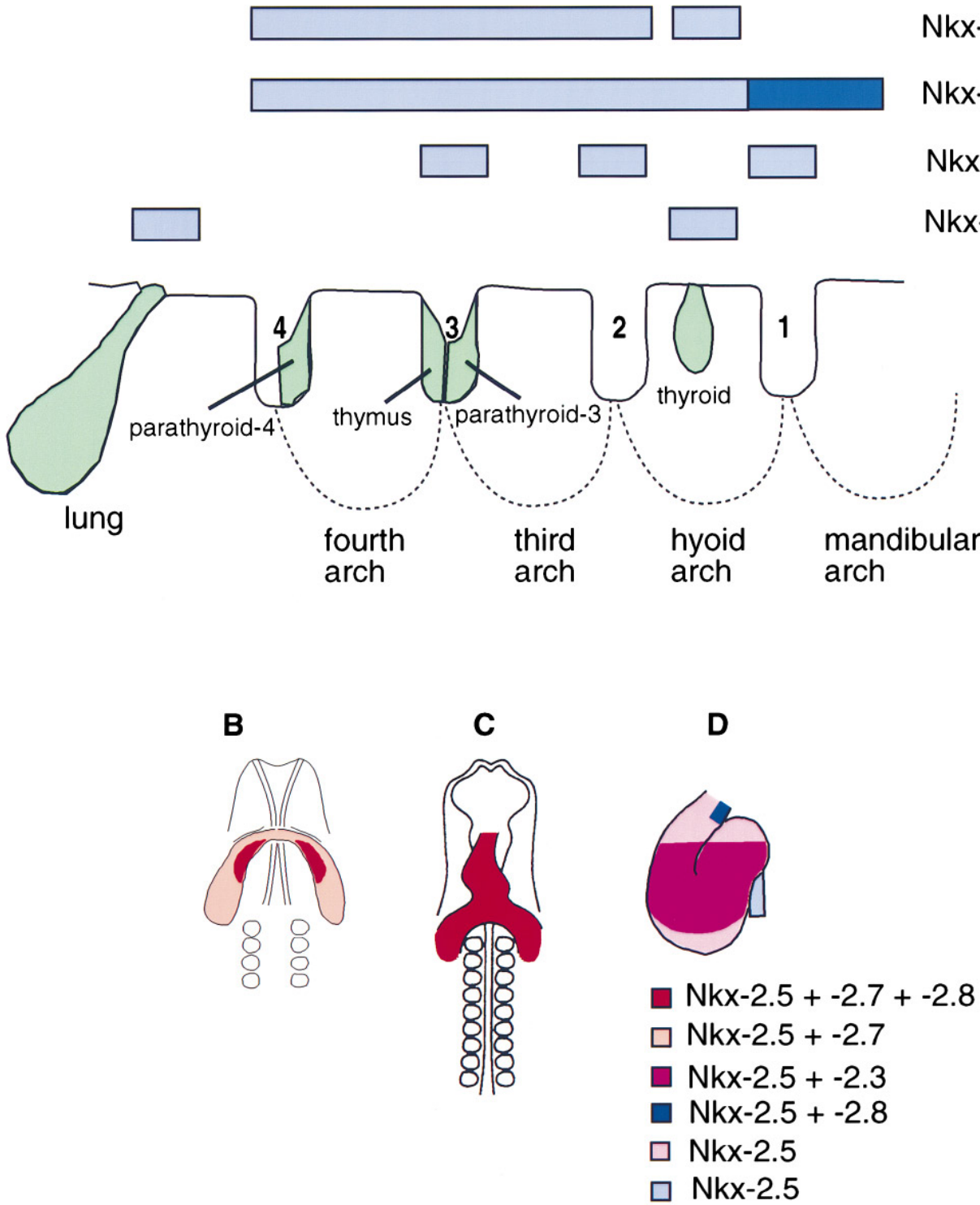

FIG. 8. Summary of Nkx expression pattern in the pharyngeal region and developing heart (see also Discussion). (A) Expression pattern of Nkx genes in the pharyngeal region. Nkx-2.3 and -2.5 are expressed in broad patterns, whereas Nkx-2.1 and -2.8 are expressed in a more localized manner. In contrast to chicken *Nkx-2.3*, zebrafish *nkx-2.3* expression extended more anteriorly, which is indicated by the deep purple box. Only one side of the pharyngeal region is depicted with the pharyngeal pouches numbered 1–4. The pharyngeal arches are indicated with dotted lines and the organ rudiments arising from the pharyngeal endoderm. (B–D) Expression pattern of Nkx genes in the developing heart. (B) HH stage 7. (C) HH stage 10. (D) Late stage. The genes that correspond to a given color are indicated at the base of D. The zebrafish *nkx-2.7* expression pattern is not known at late stages of development. Early *Nkx2-3* expression is not indicated because of species–species variation. References for expression domains are as follows: mouse *Nkx2-1* (Lazzaro *et al.*, 1991); *Xenopus*, chicken, and zebrafish *Nkx-2.3* (Evans *et al.*, 1995; Buchberger *et al.*, 1996; Lee *et al.*, 1996); mouse, chicken, *Xenopus*, and zebrafish *Nkx-2.5* (Lints *et al.*, 1993; Tonnisen *et al.*, 1994; Schultheiss *et al.*, 1995; Lee *et al.*, 1996); and zebrafish *nkx-2.7* (Lee *et al.*, 1996).

type or define a specific pattern of cardiac gene expression remains to be determined.

Nkx2-1 and *2-5* loss-of-function experiments have demonstrated the major importance of the Nkx genes for formation of the lung, thyroid, pituitary, ventral forebrain, and heart (Lints *et al.*, 1993; Kimura *et al.*, 1996). In addition, overexpression of *nkx2-5* in zebrafish embryo results in an enlarged heart (Chen and Fishman, 1996). Thus, inactivation of the Nkx genes by homologous recombination and overexpression of Nkx genes promises to address the functional significance of the expression domains and thus also of the Nkx code.

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